

USER MANUAL FOR AUTOMATED WHITEFLY NYMPHS COUNTING

NYMPHSTAR

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INTRODUCTION

Nymphstar is an open-source tool, developed in Java as several plugins for ImageJ (NIH Image). These plugins are designed to quantify the number of nymphs in third and fourth instar of the whitefly *Aleurotrachelus socialis* from photographs.

To have a proper and reliable counting, the images require a specific quality, high resolution, and light control.

System requirements:

For image acquisition is required the use a photography system which consists of these materials:

Photo Box: <http://www.or-tech.com/biobox-specs.html>

Copy stand: <http://www.amazon.co.uk/dp/B000L9QEZK>

Camera: <http://imaging.nikon.com/lineup/dslr/d300s/>

Lens: <http://www.amazon.com/Nikon-60mm-2-8G-Micro-Nikkor-Cameras/dp/B0013A1XDY>

Camera Software: http://imaging.nikon.com/lineup/software/control_pro2/

Counting software (free): <http://imagej.nih.gov/>

Installation of camera software

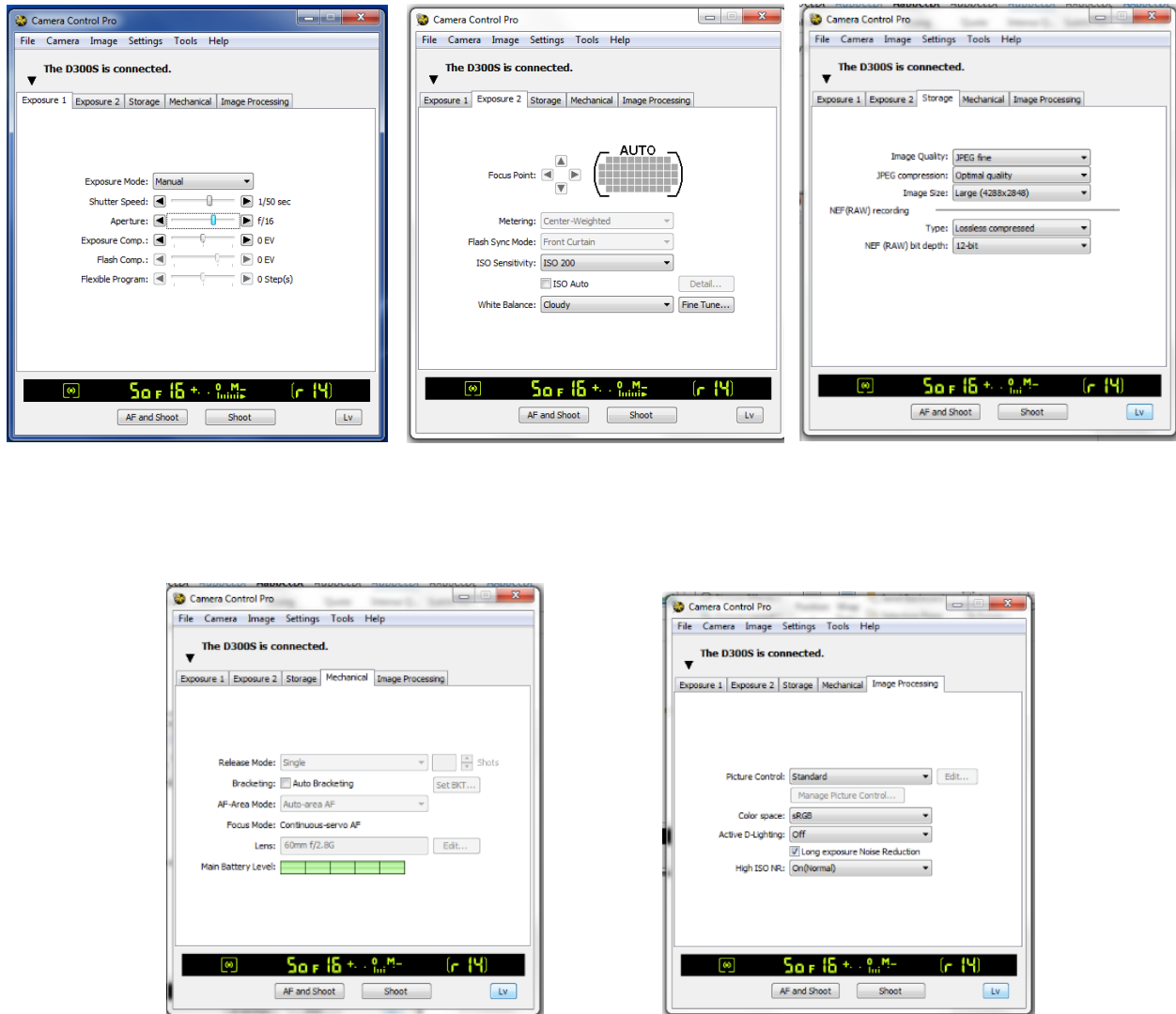
For installing the camera software Nikon Control-pro2, you have to follow the next instructions:

First, create a folder in local disk "C" and assign a desired name. Second, download "S-CCPRO_-022800WF-ALLIN-ALL____.exe" in the folder previously created. Then, execute "S-CCPRO_-022800WF-ALLIN-ALL____.exe" to start the wizard installer. Select the language and in the next window install, follow the screen instructions with the next button to end the installation.

To confirm the installation, turn off the camera and use the USB cable to connect the camera to the computer. Turn on again the camera to start the transference and then run the software.

Setting up the parameters

Once installed the camera software, is necessary set up the parameters for photographing as is shown in the next figures.



These parameters can be saved in “Settings>Save control settings” to be used later if is required, following the path “Settings>Load control settings”.

In “Tools>Download options>” is possible to change the destination folder and the numbering of the images that will be downloaded from the camera.

At the end, select the option “Lv” for a live view of the capture.

Image Acquisition

The software was developed based on an algorithm of color and size. In order to the software works well, some parameters on the pictures must remain constant, as described next:

1. Using the copy-stand, the distance between the camera and the leaf is maintained at one specific height so it is possible to compare the nymphs' number between different leaves; is it suggested a height of 70 centimeters.
2. All lights of the Photo Box must be turned on and this configuration must be constant for all the photos captured.
3. Before the photo capture and to increase the contrast, each leaf is sprayed with ethanol of 50% and with a disposable absorbent towel is removed the liquid excess.
4. Then, the leaf is placed within the Photo Box on a black background to be photographed. It could be used other color but it always must to be opaque and uniform.
5. After organize the leaf in such way that it appears complete in the screen, the capture is made clicking on the image on the surface of the leaf and then on "AF and Shoot". Automatically the camera focus the area selected and take the picture.
6. Images are stored in the folder of destination in the format JPEG previously selected with a large resolution of 4288 X 2848 pixels.

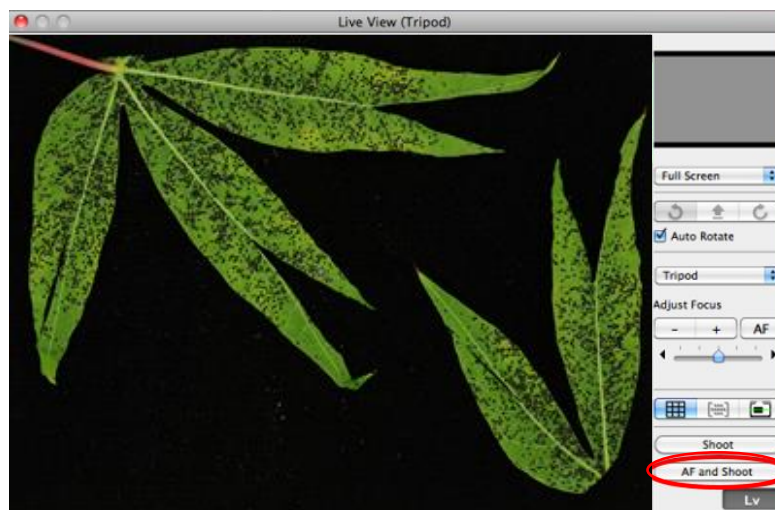


Image analysis using Nymphstar

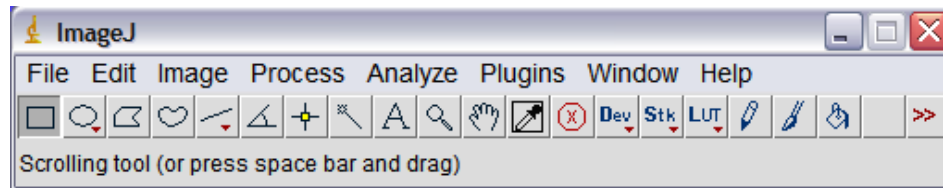
ImageJ is open-source software of public domain distributed as a portable application. That means that you do not have to run any installer.

ImageJ will run on any system that has a Java 8 (or later) runtime installed. This includes, but is not limited to:

1. Windows XP, Vista, 7 or 8 with Java installed from java.com
2. Mac OS X 10.8 "Mountain Lion" or later with Java installed from java.com
3. Ubuntu Linux 12.04 LTS or later with OpenJDK 8 installed

To download ImageJ just follow the next steps or watch this [Video tutorial to download ImageJ](#):

1. Open ImageJ download webpage in this link, << <https://imagej.nih.gov/ij/download.html>>>.
2. Select the system in which your computer works on (windows, linux, mac, etc).
3. Unpack in the local disk C the zip file downloaded "[ij152-win-java8](#)".
4. Open the Folder with the name ImageJ.
5. Double click in the application and start using it.

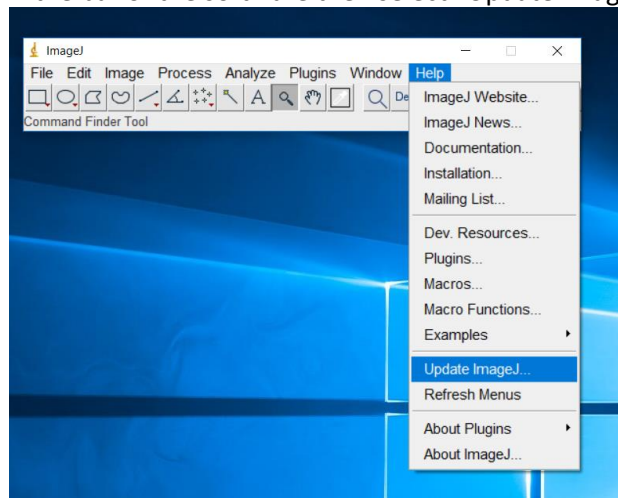


Suggestion: To access easily to the program, pin ImageJ to the start or to the task bar or create a shortcut in the desktop.

Update the version of ImageJ:

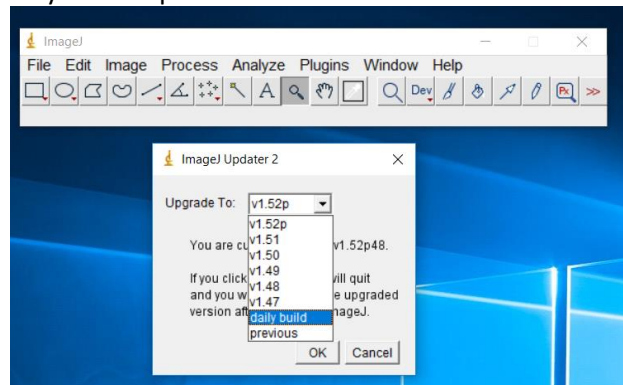
To update the version of your ImageJ is needed to follow these steps:

1. Open the application into the folder ImageJ in C, if is not opened already.
2. Go to "Help", located in the bar of the software then select "Update ImageJ...".



3. This will display a window where you should click the arrow in front of "Upgrade to:", and in the

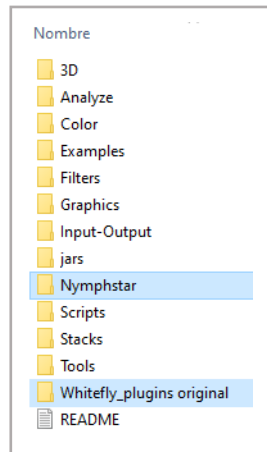
list displayed, select "daily build" option. Then click on "Ok".



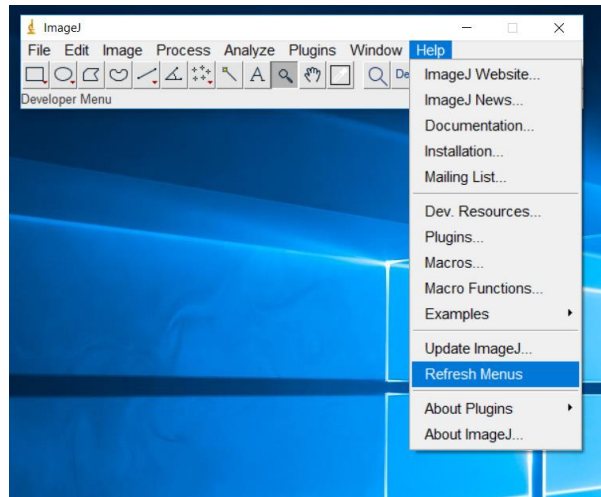
4. In seconds, the software will start to upgrade.
5. After the process ends, ImageJ will be closed automatically.

How to install the package Nymphstar

1. Starting with ImageJ closed you should extract Nymphstar from the zip file.
2. Copy the folders: "Nymphstar" (macro files) and "Whitefly_plugins_original" (program files) into the folder of **ImageJ\plugins** located in the local disk C. After made this, the file will look like this image.

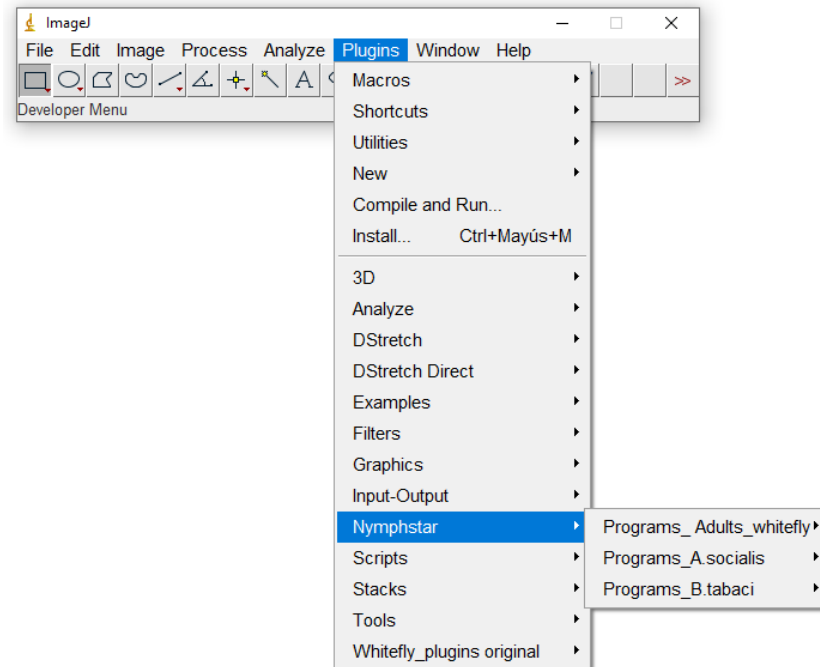


3. Then open the application ImageJ.
4. Go to option "Help", located in the bar of the software, and select "Refresh Menus"
5. In seconds, the software will be upgraded, refreshed, and ready to be used.



How to use the package Nymphstar

1. In the application ImageJ, go to the menu Plugins.
2. Then select in the menu the option Nymphstar and then “Programs_A.socialis”.



Note. It must be appearing those macros showed in the figure above. Also, in the option “Whitefly_plugins original” must appear the programs described in the appex 1.

3. Select the option you want to use to counting a single image or run “Lots analysis” for a group of images in batch: “Single Analysis Socialis” or “Lots Analysis Socialis”

For running **one picture**, the steps are as follows:

1. Click in the option: “Single_Analysis_socialis”
2. Immediately the window “Open” will be displayed.
3. In that window, you should select the picture to process and click in “Open” to end the selection.
4. Immediately the process will start and the nymphs on the image will be quantified.
5. Finally, as a result the program will give the processed image and a table with the results of the quantification.

A screenshot of the 'Results' window in ImageJ. It displays a table with the following data:

	File	Number of nymphs	Nymphs' percentage (%)	Nymphs' area (pixels)	Leaf area (pixels)
1	Sample_Protocol_1.jpg	1614	4.974	196241	3945029

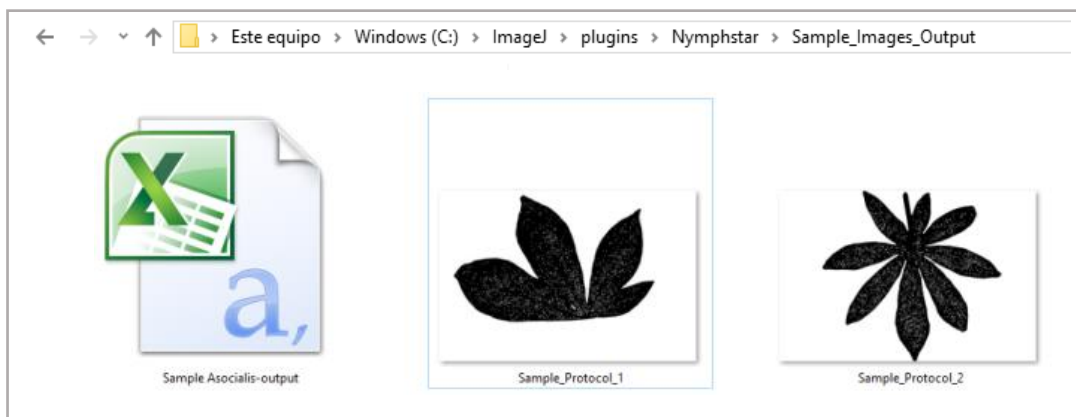


Note. In this case you decide to save the results (File\Save table) or copy them to a file.

For running a **set of pictures**, the steps are as follow:

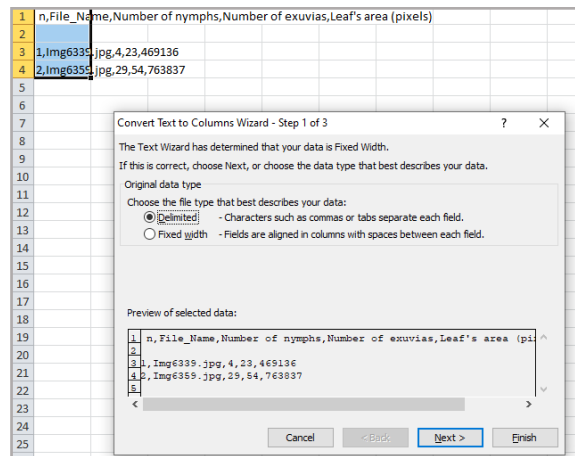
1. Click in the option: “Lots_Analysis_Socialis”
2. Immediately one window will be displayed to choose the **source** directory.
3. Now, another window is opened to choose the **destination** directory, you must create a new folder in which will be stored the processed images and the counting results.
4. Immediately will start the images processing.
5. When the analysis finishes, as a result, in the destination folder you will find the processed images and a file .csv with the results of the quantification.

Results for “Lots Analysis Socialis”



	A	B	C	D	E
1	n	File_Name	Number of nymphs	Number of exuvias	Leaf's area (pixels)
2					
3	1	Img6339.jpg	4	23	469136
4	2	Img6359.jpg	29	54	763837
5					
6					

Note. As it is a set of images, the program automatically will save the data (csv file) under the name of the folder and the processed pictures (jpg) under their original name. In some versions of Excel, is possible that you need to separate the results to columns to visualize them. Select the cells in the first column, go to “Data\Text to Columns” and choose “Delimited”.



In the next step unselect “Tap” and select “Comma” and “Finish”. At the end, data of Number of nymphs, Nymphs’ percentage (%) referred to the relation between total leaf area and the total nymphs area, Nymphs’ area and Leaf’s area in pixels are separated in columns.